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Influence of pH, salt, and temperature on pressure inactivation of hepatitis A virus ☆

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ABSTRACT

The effects of pH (3–7), NaCl (0–6%), and temperature on pressure inactivation of hepatitis A virus (HAV) were determined. The HAV samples were treated at 400 MPa for 1 min at 5, 20, and 50 °C. Decreasing solution pH enhanced pressure inactivation of HAV. This enhanced inactivation effect was most evident at 20 °C. A baroprotective effect was observed for NaCl concentrations from 1 to 6%. For example, a treatment of 400 MegaPascals (MPa) for 1 min at 50 °C reduced the HAV titers by 4.0, 4.1, 1.3 and 0.4 log plaque forming units (PFU)/ml for NaCl concentrations of 0, 1, 3, and 6%, respectively. Overall, increasing the treatment temperature enhanced pressure inactivation of HAV in the solutions. The pressure resistance of HAV in oysters was also examined. Temperature in the range of 5 to 50 °C did not significantly affect the pressure inactivation of HAV within oyster homogenates. It is concluded that HPP treatment of oysters at temperatures above room temperature would not provide any additional benefit for inactivation of HAV. However, the observation that HAV inactivation is enhanced in acidic matrices is information that may be useful for designing product formulations and processing parameters for high pressure processing of products such as low pH fruit juices and salsa.

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1. Introduction

Bivalve mollusks obtain food from the environment by filtering water, and in this process they can concentrate pathogens such as hepatitis A virus (HAV) from fecally-polluted waters. Since the linkage of hepatitis A infection to raw shellfish consumption approximately 50 years ago, HAV outbreaks associated with seafood, have been, and currently remain, a serious public health concern. While an efficacious vaccine has reduced the overall incidence of HAV in the US and elsewhere, shellfish-associated outbreaks still occur (Croci et al., 1999a; Fleet et al., 2000; Lees, 2000; Shieh et al., 2007). HAV is hardy in the environment (Bidawid et al., 2000; Cliver, 1995) and can remain infectious within oyster tissues for as long as three weeks (Kingsley and Richards, 2003). Chilling and/or freezing of shellfish after harvesting does not inactivate HAV (Lees, 2000). Depuration, i.e. placing shellfish in clean water for two- to three-days after harvesting, is commonly used to reduce the numbers of fecal bacteria in shellfish, but it does not eliminate viruses (De Medici et al., 2001; Fleet et al., 2000; Kingsley and Richards, 2003; Richards, 1988).

High pressure processing (HPP) has been used commercially in the USA for several years to facilitate the shucking of raw oysters. HPP has the additional benefit of inactivating HAV, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* in oysters (Calci et al., 2005; Cook, 2003; Kural et al., 2008; Kural and Chen, 2008), as well as viruses studied as surrogates for human norovirus (Chen et al., 2005; Kingsley et al., 2007). Consequently, HPP has strong potential as an intervention technique for inactivating food-borne pathogens associated with shellfish. For inactivation of viral pathogens by HPP, the amount of pressure applied, the treatment time, the temperature at which pressure is applied, and the nature of the environment surrounding the viruses can substantially influence the efficacy of pressure inactivation. Understanding the influence of these factors on pressure inactivation of viruses would be beneficial for formulating HPP products and identifying appropriate processing parameters. Generally, the extent to which microorganisms are inactivated increases logarithmically with increasing pressure, while a diminishing increase in inactivation, or tailing effect, is observed as treatment times increase (Chen et al., 2004; Chen et al., 2005; Kingsley et al., 2006; Kingsley et al., 2007; Kingsley and Chen, 2008). In contrast, temperature has disparate effects on virus inactivation by pressure. For the feline calicivirus (FCV) and murine norovirus (MNV), two members of the calcivirus family, inactivation increased by several log units when HPP was performed at refrigeration temperatures rather than at room temperature (Chen et al., 2005; Kingsley et al., 2007). While temperatures above 30 °C were not evaluated for MNV, temperatures of 40 °C and higher also were observed to enhance inactivation of FCV (Chen et al., 2005). For HAV, pressure inactivation was enhanced at temperatures above 30 °C as compared with inactivation at temperatures ranging from 5 °C to 30 °C (Kingsley et al., 2006).

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For substrates in which a virus is suspended, Chen et al. (2004) found that bacteriophage λ had a much higher resistance to pressure in 2%-reduced fat milk than in suspension medium (SM) buffer, which indicated that some components of the milk were baroprotective. This conclusion is supported by findings of varying inactivation of HAV by pressure in different substrates such as in Dulbecco's modified Eagle medium with 10% fetal bovine serum (DMEM-FBS), strawberry puree, green onions, and oyster meats (Calci et al., 2005; Kingsley et al., 2002; Kingsley et al., 2005). Recently, a pressure inactivation study of FCV exposed to various concentrations of NaCl and sucrose and to buffers of various pH values was performed by Kingsley and Chen (2008). A baroprotective effect was observed for NaCl and sucrose at concentrations up to 12 and 40%, respectively. Over the pH range 3–8, FCV was more resistant to pressure at ≤pH 5.2.

Since FCV is an animal virus, and only used as a surrogate for human norovirus, we extended and expanded our study to determine the effects of temperature and substrates, pH and salt, on pressure inactivation of HAV in different solutions. Previously, HAV inactivation by HPP was found to differ from inactivation of caliciviruses, in that inactivation of calcivirus was enhanced while inactivation of HAV was reduced by colder temperatures (Chen et al., 2005; Kingsley et al., 2006). Since these studies were conducted in DMEM-FBS medium, we determined whether this temperature-dependent inactivation of HAV would be observed in a real food system, oysters. Understanding the influence of these factors on pressure inactivation of viruses will be beneficial for designing product formulations and pressure processing parameters.

2. Materials and methods

2.1. Assay of HAV samples

HAV was obtained from the American Type Culture Collection (Manassas, VA, USA) as VR1402, a cell culture-adapted cytopathic clone of strain HM-175/18f. The virus was propagated on fetal rhesus monkey kidney (FRhK-4) cells. All viral stocks were stored at −70 °C in DMEM (Gibco-BRL, Gaithersburg, MD, USA) with 10% FBS (Gibco-BRL) prior to use. For plaque assay, 2 ml of virus stock or ten-fold serial dilutions of virus made with Earle's balanced salt solution (Gibco-BRL) were used to infect confluent FRhK-4 cells in 100-mm dishes, in triplicate, as described by Richards and Watson (2001). After 2 h, the plates were overlaid with DMEM medium with 5% FBS, and 1% agarose (Sigma-Aldrich, St. Louis, MO, USA). At 14–17 days post inoculation at 37 °C, HAV was inactivated by 5% formalin treatment, the agarose overlay was removed, and HAV plaques were visualized by crystal violet (Fisher Scientific, Hampton, NH, USA) staining.

2.2. Pressure inactivation of HAV suspended in different pH and salt solutions

Ten ml volumes of HAV in DMEM-FBS were dialyzed using 14,000 MW cutoff dialysis tubing (SpectraPor, Rancho Domingo, CA) against 1 L of 0.1 M solutions of HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; Invitrogen) at pH 3.0, 4.0, and 7.0 or 0.1 M MES (2-4-morpholino-ethane sulfonic acid; Fisher) at pH 5.2 and 6.0 at 4 °C overnight. Buffers were changed three times. These buffers were chosen because their pH values do not change substantially under high pressure (Kitamura and Itoh, 1987).

HAV stock in DMEM-FBS and NaCl (6% w/v; Fisher) were made by dissolving salt within the virus stock followed by 0.22-μm filtration. The HAV stock was then mixed proportionally with HAV stock in DMEM-FBS with 6% NaCl to generate concentrations of 0.15, 0.3, 1.0, 3.0, and 6.0% (w/v). HAV in DMEM-FBS without additional NaCl was used as a control. It is important to note that DMEM-FBS alone contains 0.5–0.7% (w/v) NaCl.

One ml of HAV samples suspended in buffers with different pH values or in different NaCl solutions was transferred into polyester Scotchpak pouches (Kapak 500, Minneapolis, MN) with a second pouch sealed around the first pouch. Heat-sealing was performed using an Impulse Food Sealer (Model MP-8; American International Electric, Whittier, CA; USA). Sample pressurization was conducted using a high pressure unit with temperature control (Model Avure PT-1, Avure Technologies, Kent, WA) using water as the hydrostatic medium. A circulating water bath surrounding the pressure cell controlled the temperature of the pressure chamber. Temperatures of the water bath and samples inside the chamber during pressurization were monitored using K-type thermocouples. The temperature and pressure data were recorded every 2 s (DASYTEC USA, Bedford, NH, USA). The samples were treated at 400 MegaPascals (MPa) for 1 min at initial sample temperatures of 5, 20, and 50 °C. The pressure come-up rate was 22 MPa/s and pressure-release time was <4 s. Pressurization time reported in this study did not include the pressure come-up or release times. Temperature increases during pressure treatment due to adiabatic heating were 2.1, 2.5, 3.3, and 3.8 °C/100 MPa at initial sample temperatures of 10, 20, 40, and 50 °C, respectively (Chen et al., 2005). The titers of the treated and untreated samples were determined as described above.

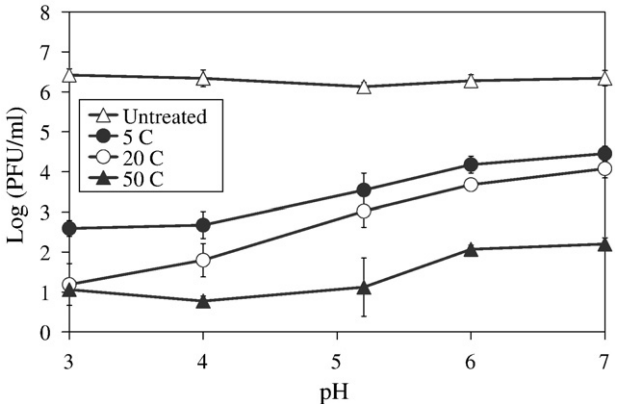

Fig. 1. Effect of pH on pressure inactivation of hepatitis A virus in HEPES or MES buffers. HEPES buffers were used for pH 3.0, 4.0, and 7.0, MES buffers for pH 5.2 and 6.0. Samples were treated at 400 MPa for 1 min at 5, 20, and 50 °C. Data are the means of 3 independent trials. Error bars represent ±1 standard deviation.

2.3. Pressure inactivation of HAV within an oyster homogenate

Live Eastern oysters (*Crassostrea virginica*), obtained from the College of Marine Studies at the University of Delaware, Lewes, DE, were shucked and meats were homogenized for 180 s in a blender (Waring model number 31BL91, New Hartford, CT, USA). Two ml of HAV in DMEM-FBS was inoculated into approximately 100 g of oyster meats to obtain a final concentration of approximately 4×10^4 PFU/g and mixed well. Portions of 5 g of blended meats were placed into individual plastic pouches and heat-sealed as described above. Oyster samples were treated at 350 MPa for 2 min, 400 MPa for 1 min, 450 MPa for 1 min, or 500 MPa for 1 min. The initial sample temperatures before pressurization were 20, 40, and 50 °C. The titers of the treated and untreated samples were determined as described above. The pH of the uninoculated blended oyster meats was measured using a pH meter (Model AR-50; Acumet Research, Fisher).

2.4. Statistical analyses

Three independent trials were conducted for all pressure treatments. Statistical analyses were conducted using Minitab® Release 15 (Minitab Inc., University Park, PA, USA). One-way analysis of variance (ANOVA) was used to compare significant differences ($P<0.05$) between different treatments. A General Linear Model was used to determine the main effects of pH and temperature and their

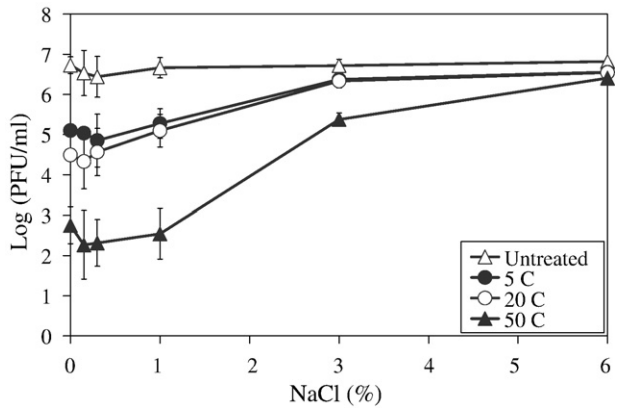

Fig. 2. Effect of NaCl concentration (0–6%, w/v) on pressure inactivation of hepatitis A virus (HAV). HAV was suspended in DMEM-FBS and different amounts of NaCl. Samples were treated at 400 MPa for 1 min at 5, 20, and 50 °C. Data are the means of 3 independent trials. Error bars represent ±1 standard deviation.

interaction effect as well as NaCl and temperature and their interaction effect.

3. Results and discussion

The pH effect on the pressure inactivation of HAV is shown in Fig. 1. HAV samples were dialyzed against 0.1 M buffers with pHs ranging from 3 to 7. Without pressure treatment (untreated samples), HAV was very stable within the pH range of 3 to 7 with titers varying from 6.1 to 6.4 log PFU/ml. The pH stability of HAV observed is consistent with its reported properties (Scholz et al., 1989). An acidic environment (low pH) significantly enhanced pressure inactivation of HAV at the three temperatures tested ($P<0.05$). This enhanced pressure inactivation effect was the most evident at 20 °C, with a 2.9 log difference in titers between pH 3 and pH 7 ($P<0.05$). The colder temperature (5 °C) significantly reduced HPP inactivation throughout the pH range, while the higher temperature (50 °C) showed enhanced inactivation by HPP ($P<0.05$). This is consistent with results reported previously for HAV in DMEM-FBS (Kingsley et al., 2006) where enhanced inactivation was observed at higher temperatures and reduced inactivation was observed at colder temperatures. Statistical analyses indicated that the interaction between pH and temperature was significant ($P<0.05$). The temperature effect on pressure inactivation of HAV depended on the pH levels. For example, increasing the temperature from 20 °C to 50 °C significantly enhanced the pressure inactivation of HAV at pH 7 (1.9-log increase), but not at pH 3 (0.1-log increase).

The effect of NaCl on the pressure inactivation of HAV is shown in Fig. 2. Without pressure treatment (untreated samples), HAV in DMEM-FBS was stable when the solution was supplemented with 0–6% NaCl. The NaCl did not provide any significant protective effect for HAV against pressure for concentrations up to 1% ($P>0.05$); however, higher salt concentrations were baroprotective. The baroprotective effect of 6% NaCl was substantial enough that the most extreme treatment, 400 MPa for 1 min at 50 °C, only reduced the HAV titer by 0.4 log PFU/ml when 6% NaCl was supplemented as compared to an inactivation of 3.9 logs without NaCl supplement. The reduced inactivation of HAV in the presence of NaCl is consistent with results reported for FCV (Kingsley and Chen, 2008) and in general agreement with the preliminary results characterizing HAV inactivation by HPP (Kingsley et al., 2002). The baroprotective effect of NaCl has also been reported for bacteria, such as *Lactococcus lactis* (Molina-Hoppner et al., 2004) and *Bacillus stearothermophilus* spores (Furukawa and Hayakawa, 2000). The mechanistic basis for this protective effect is not known but may relate to NaCl preferentially interacting with hydrophilic native conformations of viral proteins, altering the qualities of the aqueous solvation cage around the virus proteins, stabilization of void volumes by displacing water molecules or, altering the solution density and compressibility under pressure. Temperature significantly affected pressure inactivation of HAV ($P<0.05$). The effect of interaction between NaCl and temperature was significant ($P<0.05$), indicating that the effect of temperature on pressure inactivation of HAV depended on the NaCl concentrations. For example, increasing temperature from 20 °C to 50 °C significantly enhanced pressure inactivation of HAV at a supplemented NaCl concentration of 0% (1.7-log increase), but not at supplemented NaCl concentration of 6% (0.1-log increase).

Since pH, salt, and temperature affected the pressure inactivation of HAV, it was desirable to investigate the effectiveness of HPP on the inactivation of HAV in an oyster matrix. Previous work demonstrated that HAV could be directly inactivated within whole in-shell oysters by HPP (Calci et al., 2005). Subsequent study showed that warm temperatures enhanced inactivation of HAV in DMEM-FBS (Kingsley et al., 2006) and since the previous oyster work was performed at an initial sample temperature of 9 °C (Calci et al., 2005), investigation of the potential for enhanced inactivation within an oyster matrix at elevated temperatures was warranted. Results for pressure inactivation of HAV in oysters are shown in Fig. 3. As expected, when the treatment pressure increased, the inactivation of HAV was enhanced. In contrast to the findings on temperature effect observed previously in buffers and salt solutions, slightly elevated temperature did not significantly enhance pressure inactivation of HAV ($P>0.05$) in an oyster homogenate. The reason for the non-enhanced inactivation of HAV by HPP at higher temperatures is unknown, but presumably the observed resistance is due to the composition of oysters that mitigates the combined inactivation by pressure and high temperature. The pH of oyster homogenate was 6.07 and oysters are reportedly composed of 5.5% carbohydrate, 0.178% sodium, 4.8% protein, and 1.2% fat (USDA National Nutrient Database, 2008). When the results for HAV in oyster homogenates (Fig. 3) were compared with those for HAV at pH 6 in 0.1 M MES (Fig. 1) and those for HAV in 0.15% and 0.3% NaCl solutions (Fig. 2), it was clear that HAV was more resistant to pressure in oyster homogenates than in NaCl solutions and the MES buffer, suggesting that some oyster components are baroprotective.

Temperature increases during pressurization due to adiabatic heating. With an initial sample temperature of 50 °C, the highest temperatures reached by the oyster samples during pressurization were 63 °C at 350 MPa, 65 °C at 400 MPa, 67 °C at 450 MPa, and 69 °C at 500 MPa. At atmospheric pressures, these elevated temperatures approach thermal inactivation temperatures. To evaluate the potential of a thermal component for pressure inactivation, HAV stocks in DMEM-FBS were incubated for 5 min at 63, 65, and 67 and 69 °C.

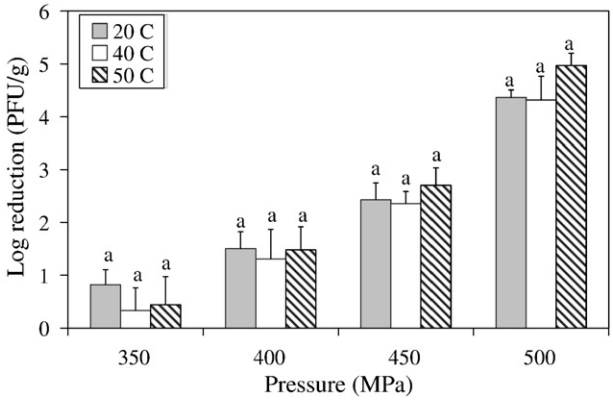

Fig. 3. Pressure inactivation of hepatitis A virus (HAV) in oysters. Pressure treatments were carried out at 350 MPa for 2 min, 400 MPa for 1 min, 450 MPa for 1 min, and 500 MPa for 1 min. Data are the means of 3 independent trials. Error bars represent ±1 standard deviation. Log reductions = log (titers of untreated samples) − log (titers of treated samples). At each pressure, columns labeled with the same letter are not significantly different ($P>0.05$).

Observed log reductions were 3.3, 3.3, 5.0, and 4.6, respectively, indicating adiabatic heating may have contributed to pressure inactivation observed at the 50 °C initial sample temperature. However, it should be noted that treatment of HAV-contaminated mussels at 60 °C for 30 min or 80 °C for 10 min was reportedly not sufficient to completely inactivate HAV in mussels (Croci et al., 1999b).

While the application of elevated temperatures for inactivation of HAV within oysters by HPP does not appear to offer much benefit, the finding that acidic pH significantly enhanced pressure inactivation of HAV can be used to design product formulations for pressure processing and is of potential benefit for processing acidic fruit and vegetable products. Results obtained here both validate, and confirm, the enhanced sensitivity observed previously with HAV-contaminated sliced green onions and strawberry puree that had the pH values of 5.12 and 3.67, respectively (Kingsley et al., 2005). One potential HPP food application is salsa, an acidic food with pH typically ≤5. This food was implicated in an HAV outbreak due to contaminated green onions that resulted in 555 cases and 3 deaths in 2003 (Center for Disease Control (CDC), 2003).

Mechanistically speaking, it is unclear why acidic pH enhances HPP inactivation of HAV. For two other picornaviruses, loss of a viral capsid protein VP4 has been associated with inactivation by HPP (Gonçalves et al., 2007; Oliveira et al., 1999). Whether this is also the case for HAV remains to be determined. For pH-dependent enveloped viruses, it is known that high pressure can trigger conformational changes in surface glycoproteins associated with membrane fusion and virus entry in lieu of low pH application. Although HAV is not a pH-dependent enveloped virus, as part of the infectious process, the HAV capsid must undergo low pH-triggered conformational changes after entering the acidic endosome/lysosome to release its RNA into the cytosol of the cell and initiate replication. Thus synergistic effects of both pressure and acidic pH are not unprecedented for viruses. This acidic pH pressure effect contrasts with results obtained for FCV which was more resistant to pressure at ≤pH 5.2 (Kingsley and Chen, 2008) and results reported for the tobacco mosaic virus (Santos et al., 2004). The reason for this different behavior in response to pH is unknown. It is also worth noting that the pH effect for pressure inactivation of HAV was generally in agreement with results reported for bacteria (Alpas et al., 2000; Stewart et al., 1997). For example, results reported for *Salmonella* Typhimurium (pH 5.6 versus pH 7.0) and *E. coli* O157:H7 (pH values of 3.4–4.5 versus pH 5.0) have shown that lower pH values enhance inactivation by high pressure (Linton et al., 1999; Ritz et al., 1998).

In light of this study, we recommend that room temperature be used for high pressure processing of oysters potentially contaminated with HAV. The finding that low pH substantially increases pressure inactivation of HAV can be used for the application of HPP in acidic fruit and vegetable products.

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